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1

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The purpose of this work is to determine whether genomic instability following radiotherapy is increased in breast cells that harbor either a BRCA-1 or BRCA-2 mutation relative to cells which carry two normal copies of the gene. Therefore mammary epithelial cells from women at high risk for carrying either a BRCA-1 or BRCA-2 germline mutation will be compared with mammary cells from women not at risk for carrying a mutation following radiation using the following assays: growth factor independence, growth in soft agar, and tumorigenicity in vivo. The work done thus far has largely focused on characterizing the high-risk cell lines. All baseline radiation experiments have been done and the growth factor experiments have begun, which show a dependence upon EGF in unirradiated high risk (and control) cell lines. Cells have also been infected with either HPV E6 and E7 to delay senescence and conduct comparison studies between cell lines with or without a BRCA mutation.

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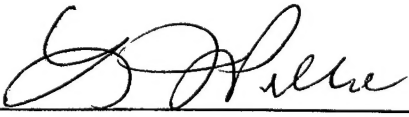
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3

Lori Pierce, M.D.

The Effect of Radiotherapy Upon Primary Human Mammary Epithelial Cells Which Harbor a Breast Cancer Susceptibility Gene

Table of Contents

Annual Report
DAMD17-96-1-6194

The Effect of Radiotherapy Upon Primary Human Mammary Epithelial Cells
Which Harbor a Breast Cancer Susceptibility Gene

Introduction

Inherited breast cancers account for 5 to 10% of all breast malignancies, and cancers associated with mutations in BRCA-1 and BRCA-2 are estimated to account for approximately 45% of the cases of hereditary breast cancer. The functions of the BRCA-1 and -2 genes are unknown. Recent data, however, suggest that both may be involved in DNA repair (1,2). Using an embryonic expression pattern of the mouse BRCA-2 gene, a direct interaction between the BRCA-2 protein and the DNA-repair protein MmRad 51 (the mouse homologue of Rad 51) has now been identified (1). This suggests that BRCA-2 is an essential cofactor in the Rad-51-dependent DNA repair of double-strand breaks. Studies by Sharan et al. also demonstrated that embryonic mouse cells in which the murine version of BRCA-2 had been inactivated could not recover from gamma radiation damage (1). Colocalization and coimmunoprecipitation studies have also shown that the BRCA-1 protein interacts indirectly with HsRad 51, a human homolog of bacterial RecA (2). This suggestion of a functional interaction between the two proteins may mean that BRCA-1 is involved in the maintenance of genomic integrity and perhaps DNA repair mechanisms of the cell.

Concerns have therefore been raised as to whether these cancer-predisposing genes have an enhanced radiosensitivity compared to breast cells without a breast cancer susceptibility gene mutation. Therefore, this grant will examine whether there is an increased susceptibility to radiation-induced transformation in mammary epithelial cells which either are known to have a BRCA-1 or 2 mutation or are from patients at high risk for having a mutation, and compare the results with those obtained from cells from patients not at risk for carrying a mutated breast cancer susceptibility gene.

The goal of this work is to determine whether breast cancer predisposition is correlated with enhanced cellular sensitivity to radiotherapy. Enhanced sensitivity to radiation damage will be assessed by determining whether immortalized irradiated cells from high risk patients are more likely to exhibit growth factor independence and anchorage-independent growth compared to cells without a breast cancer susceptibility gene, and to observe for any differences in rates of

tumorigenicity *in vivo* following radiotherapy compared to irradiated cells that do not carry a germline mutation.

Body

The initial aims of this grant are to determine whether cells from patients at high risk for harboring a germline mutation for a breast cancer susceptibility gene are more susceptible to immortalization following therapeutic doses of radiation *in vitro* compared to cells from reduction mammoplasty specimens from patients not at risk for hereditary breast cancer, and to determine whether immortalized irradiated cells from patients at risk for a germline mutation were more likely to exhibit growth factor independence and anchorage-independent growth relative to immortalized cells from patients not at risk for carrying a breast cancer susceptibility gene.

As designated in the grant, cell lines 6-16, 7-31, and 4-12 have been taken from patients at high risk for carrying either a BRCA-1 or 2 mutation. Although 4-12 was shown, using single stranded conformation polymorphism (SSCP) testing, to have a mutation at exon 24 of the BRCA-1 gene, subsequent sequencing of this mutation did not reveal a known functional mutation, i.e, was not associated with a truncated protein. Therefore, the genetic alteration detected via SSCP was conservatively assumed to be a polymorphism. Additional cell lines were later established from women, who by family history, were at high risk for carrying either a BRCA-1 or 2 mutation. These are denoted as 11-21, 5-24, and SUM 194. The results of experiments with these cells are compared with parallel experiments done with cell lines from patients with no family history of female or male breast cancer or ovarian cancer (i.e, at minimal risk for carrying a BRCA-1 or 2 mutation). These control cell lines are named 1-30, 1-24, and 11-6.

After extensive testing using conformation sensitive gel electrophoresis (CSGE) followed by direct sequencing during the first year of the grant, we now know that both cell lines 4-12 and 11-21 carry a germline BRCA-1 mutation. Specifically, 4-12 has a mutation 5382 InsC in exon 20 of the BRCA-1 coding sequence which is a frameshift mutation and leads to a premature stop codon at amino acid 1829 and a truncated BRCA-1 protein. The cell line 11-21 has a single base substitution C4302 to T4302 in exon 12 of BRCA-1. This mutation gives rise to a nonsense mutation and a prematurely truncated BRCA-1 protein. Thus 4-12 and 11-21 were used for subsequent experiments as the cell lines heterozygous for a BRCA-1 mutation. Due to the extreme limitation in number of the 4-12 HME mammary epithelial cells that were available for research, all cells were infected with E6 and E7 so that they could be maintained in culture indefinitely. Therefore, all experiments done on BRCA-1 HME heterozygote cells were done on 11-21 HME.

6

We have conducted the baseline experiments, as designated in the Statement of Work (SOW). The doubling times of the non-irradiated mammary epithelial cell lines were determined. This was done with the intent to establish a baseline for comparison with irradiated cell lines. The doubling times varied significantly by passage number within each cell line, quantifying progression to senesence. For example, the doubling time for cell line 7-31 passage (P)1 was 52.9 hours, P2 77.6 hours, and P3 136.6 hours; corresponding doubling times for 6-16 were 52.3, 64.8, and 178.4 hours, respectively; and 66.5, 70.3, and 123.8 hours for cell line 11-21. Passage 1 cells were not available for control line 1-24; however, P2 and P3 times were 66.9 and 95.9 hours, respectively. Because doubling time varied so significantly by passage number, we were not able to use doubling time as a reliable early indicator of emerging genomic differences between irradiated and unirradiated cells. Baseline karyotypes were done of 11-21 HME (with the known BRCA-1 mutation) and its control cell line 11-6 HME. In the baseline karyotype of 11-21, five normal cells were seen, one cell was diploid with an add(8)(q). A second cell had 84 chromosomes with additional material on one of the three chromosomes 2, a deleted 7q and the add (8q). The karyotype of HME 11-6 consisted of mostly normal cells with some random non-clonal changes.

Baseline clonogenic survival assays were performed on the BRCA-1 cell line 11-21HME. The 3 control cell lines (11-6, 1-24, and 1-30) were also compared. Single doses of 2, 4, 6, 8, 10, and 15 Gy were used. Cell survival curves were superimposable for 11-21HME and the low-risk curves. For all cell lines irradiated, the dose of radiation resulting in approximately 10-15 % cell kill was 5 Gy. Thus, 5 Gy was chosen as the dose to be used for our radiation experiments in addition to the fractionation regimen used by Wazer, et al of 30 Gy in 2 Gy fractions, previously associated with malignant transformation of a mammary cell line (3).

We then irradiated 11-21 HME and 11-6 HME cells after they were plated at approximately 70-80% confluency using 5 Gy as a single dose and the 30 Gy in 2-Gy fraction regimen. Cells where then maintained and observed for 3 months. We did not observe any evidence of spontaneous immortalization of either the BRCA-1 or control mammary epithelial cell lines. All cells senesced at or between passages 4-6. Thus, there have been no clones to re-karyotype.

Because normal mammary epithelial cells do senesce in culture in early passage, as previously shown, we infected the HME cells with the E6 and E7 genes from human papilloma 16 virus, which, in most cells, immortalize them in culture. While this removes spontaneous immortalization as an endpoint in this study for these cells, it allows on-going comparisons between wild-type and heterozygote BRCA-1 cells for

other endpoints. We performed the baseline karyotypes on unirradiated 11-21 HPV (infection with both E6 and E7) cells and control cell line 1-30 HPV. There were considerable karyotypic changes associated with the HPV infection including 45-47,XX,der(4)t(1;4)(p13;q21),iso(8)(q10),+iso(9)(q10) in the 1-30 cells and 47,XX,iso(q)(q10),iso(9)(q10) for the 11-21 HPV cells. Thus karyotypic changes would be difficult to follow after radiotherapy for the HPV infected cell lines. Doubling times were, however, relatively consistent for HPV cell lines unlike the HME cells. Doubling times for were approximately 33 hours for the 11-6 HPV unirradiated controls versus 31 and 45 hours for the 4-12 HPV and 11-21 cells, respectively.

Because it has been shown that human breast cancer cells express cytokeratin markers consistent with an origin from the luminal cells of the terminal ductal lobular unit, immunocytochemical analysis with antibodies specific for the luminal marker keratin-19 (K-19) was performed on cell lines 1-30 (HPV), 4-12, 11-21, 11-6, and 1-24 to insure that cells of the appropriate lineage for malignant transformation were present in culture. All cell lines were K-19 positive.

Since growth factor independence has long been recognized to play an important role in tumor development and progression, one of the aims of the grant is to compare growth factor independence in irradiated high risk cell lines to control cell lines as an indication of increased tumorigenicity. In preparation for these experiments, the growth factor requirements of the unirradiated HPV cell lines were established. All cells were grown in serum-supplemented media 5% IHEC (Ham's F-12 medium supplemented with insulin, hydrocortisone, epidermal growth factor (EGF), cholera toxin, and 5% fetal calf serum). The growth requirements of 4-12, 1-30, and 11-6 (HPV) were tested by plating cells in the following serum depleted medias: SFIHEC (serum-free media supplemented with insulin, hydrocortisone, epidermal growth factor, and cholera toxin), SFIHC (SFIHEC minus epidermal growth factor), SFHEC (SFIHEC minus insulin), SFIHE (minus cholera toxin), and SFIH (minus epidermal growth factor, cholera toxin). Cell counts in each of the serum-depleted medias were comparable to those in 5% IHEC in the first passage. We next looked at the growth requirements in passage 2 to determine the requirement for EGF. Cells were grown in SFIHC and each cell line demonstrated increasing cell death with increasing time in culture. Therefore, infection with E6 and E7 did not confer EGF independence. Thus, development of EGF independence would be a marker of phenotypic changes between the BRCA-1 heterozygote and wild-type cells.

Clonogenic cell survival curves were also performed on the HPV-immortalized cells using the same RT doses as previously described. Just

as shown in the HME cells, 5 Gy as a single fraction resulted in approximately 10-15 % survival (Figure 1).

Soft agar assays were performed on the unirradiated HPV cells to assess growth. Unlike the HME cells which are unable to grow in soft agar, the cells infected with both E6 and E7, with or without the BRCA-1 mutation show extensive growth in soft agar. Thus, soft agar growth was removed as a selectable phenotype between cells with and without a germline mutation.

Studies of irradiated 11-21 HPV and 11-6 HPV (control) cells are currently on-going to look for EGF independence. Because we previously demonstrated the dependence of the unirradiated HPV cells on EGF, we have irradiated 11-6 HPV and 11-21 HPV with 5 Gy and are subculturing the cells in media with and without serum and with and without EGF. At present, we have cells in 5% IHEC and SFIHEC at passages 7 and 8 following radiotherapy which are growing rapidly and to confluency, and appear robust. This compares to cells that have been subcultured in SFIHE, SFIHC, and SFIH which, despite the same period of time, are only in passage 3 or 4. Thus, no cells have emerged as EGF independent but we continue to follow them in culture.

Much of the work outlined for year 2 in the SOW cannot be completed due to the lack of emergence of either a spontaneously immortalized clone or a growth-independent clone. To establish cell lines that may have intermediate genomic changes between the HME and HPV cells and may allow additional endpoints for study, we have also infected the 11-21 and 11-6 controls with E7 alone. This infection has resulted in rapidly growing cells which have been passed in culture until approximately passage 10 prior to senescence. These cells look morphologically like normal mammary epithelial cells and do not grow in soft agar. Doubling times for these cells are intermediate between HME and HPV cells, with times for 11-21 E7 and 11-6 E7 cells of 55 hours and approximately 58 hours, respectively. We have shown that 5 Gy results in 10-15% cell survival (Figures 2 and 3) and that these cells are generally EGF dependent (Figures 4 and 5). We now have experiments on-going with 11-21 E7 cells and 11-6 E7 cells that have been irradiated with 5 Gy and other cells that have been irradiated with 30 Gy in 2 Gy fractions over 3 weeks that have been in culture for about 2 months. No immortalized clones have emerged. These irradiated cells have also been tested and are negative for soft agar growth. These cells are currently being subcultured in various depleted medias. We will continue to look for EGF-independent growth and will perform sequential flow analyses for suggestions of emerging genetic differences.

During the remaining 8 months allotted on the extension of the grant award, the E7 experiments will be completed, and any clones that may emerge will be tested in the nude mouse model.

While there have been some problems with completing all of the endpoints as outlined in the SOW as discussed above, the data that has been generated thus far does not show any suggestion of genomic instability in cells which are heterozygote for a BRCA-1 mutation compared to cells carrying two normal copies. Our preliminary data would suggest that the presence of a single copy of the gene provides genetic stability comparable to the wild-type cell.

Detailed Methods

Preparation of normal mammary epithelial cells: Breast tissues are minced with scalpels and incubated overnight in Medium 199 containing type III collagenase at a concentration of 200 units/ml and Dispase at 1mg/ml. Twenty ml of media/gram of tissue are used. The tissues are agitated gently in a shaking water bath at 37°C. The cells are then washed extensively in Medium 199, and an aliquot of the cells is counted by isolating and counting nuclei with a Coulter counter.
Excess cells from patient specimens not used in primary culture experiments are cryopreserved for future use. To freeze cells, they are suspended in Medium 199 supplemented with 20% FBS and 5% dimethylsulfoxide at a concentration of 5×10^6 cells /750 ul of freezing medium. The cells are cooled slowly to -80°C using a step freezer and then stored over liquid nitrogen.

Cell culture: Mammary epithelial cells isolated by the methods described above are seeded onto collagen-coated (Vitrogen100, Collagen Corp.) 60-mm or 35-mm culture dishes at densities ranging from 10^5 to 5×10^5 cells/dish. The complete serum-containing medium consists of Ham's F-12 (Hazelton Biologicals) supplemented with insulin (5 ug/ml), hydrocortisone (1ug/ml), epidermal growth factor (10ng/ml), cholera toxin (100 ng/ml), 5% fetal bovine serum, Gentamycin, and Fungizone.

To characterize specific growth factor requirements for proliferation of irradiated cells, a defined serum-free media will be used which contains Ham's F-12 supplemented with bovine serum albumin (1mg/ml), sodium selenite (50ng/ml), triiodothyronine (50ng/ml), Gentamycin, Fungizone with or without insulin, hydrocortisone, epidermal growth factor, and cholera toxin in the same concentrations as noted above.

Cells are maintained at 37° in a humidified atmosphere of 90% air and 10% CO_2. For subculture of cells grown in primary culture, cells are rinsed with calcium and magnesium-free Hanks' balanced salt solution and incubated with trypsin-EDTA for 2-5 minutes. The cells are reseeded at split ratios of 1:3 to 1:10.

<u>Immortalization with E6 and E7 from human papilloma 16 virus:</u> PA317 amphotrophic packaging cell lines stably transfected with pLXSN (vector), pLXSN16E6 (HPV-16 E6), or pLXSN16E7 (HPV-16 E7) with a G418 selectable marker (provided by V. Band, Tufts University School of Medicine) were grown to 70-80% confluence, and supernatants are collected for 16 hours and stored in aliquots at -80°C. Approximately 10^5 cells are plated for 25-cm^2 flask for 18 hours and infected with 100-200ul of virus stock in 2 ml of medium containing Polybrese (Sigma) at 4ug/ml for 8 hours.

<u>Differential cell isolation:</u> Filter-selection methods are used to isolate individual colonies from primary cultures derived from normal or neoplastic tissues. Cells are seeded at low density (< 10^5 cells per 60 mm dish), grown for 10 days, and then chosen for filter selection. For filter selection, small pieces of Whatman paper are soaked in trypsin-EDTA and then placed on individual colonies and incubated. Filters are then removed and placed in a 35-mm wells containing fresh growth medium. The plates are incubated overnight during which the cells detach from the filter and attach to the substratum. Cells arethen grown to confluence and subcultured.

<u>Exponential cell growth:</u> Doubling times for each of the cells will be determined as follows: 1 x 10^4 cells will be seeded in 35-mm 6-well plates. Cell nuclei will be enumerated using the Coulter counter at 2,6,10,14 and 18 days. All measurements will be made in triplicate.

<u>Radiation cell survival:</u> Irradiations will be performed at room temperature at 1.5 Gy/min using a Cobalt-6 source (AECL Theratron 80). Dosimetry calibration is carried out using an ionization chamber (Baldwin Farmer) connected to an electrometer system that is directly traceable to a National Bureau of Standards calibration. After radiation treatment, cells are assessed for clonogenic survival: Cells will be removed from the dishes with phosphate buffered saline containing trypsin-EDTA and diluted into culture dishes in numbers to yield between 20 and 200 colonies per plate. After 10-14 days, the plates were fixed with methanol-acetic acid, stained with crystal violet, and scored for colonies containing more than 50 cells. Radiation survival data will be corrected for plating efficiency using an

unirradiated plate of cells. Cell survival curves will be fit using the linear-quadratic equation (4). Radiation sensitivity is expressed in terms of the mean inactivation dose, which represents the area under the cell survival curve. The mean inactivation dose will be calculated by linearizing the linear quadratic equation through use of a logarithmic transform, fitting alpha and beta by linear regression, and integrating over this curve using the method described by Fertil et al (5). Establishment of these curves will then allow us to select a dose of radiation resulting in 10 to 50% cell survival.

Conclusions

The work done thus far has largely focused on identifying cell lines with a BRCA-1 mutation and characterizing their growth. All baseline radiation experiments have been done, showing that clonogenic survival is equivalent for the low-risk (control) and high-risk cell lines and that the dose resulting in approximately 10% survival in the HPV-infected cell lines is 5 Gy. Growth factor experiments done thus far have shown an EGF dependence in E6 plus E7 infected cells as well as cells infected with E7 only. Soft agar assays have been done in E6 plus E7 cell lines showing colony formation and E7 only cell lines which did not result in growth.

Therefore we are currently doing radiation experiments with 11-21 and control cell lines using both a single fraction (5 Gy) and fractionated radiotherapy (30 Gy in 2 Gy fractions) to look for immortalization, EGF independence, and soft agar growth, with plans to study *in vivo* tumor growth in nude mice any genomically unstable clones.

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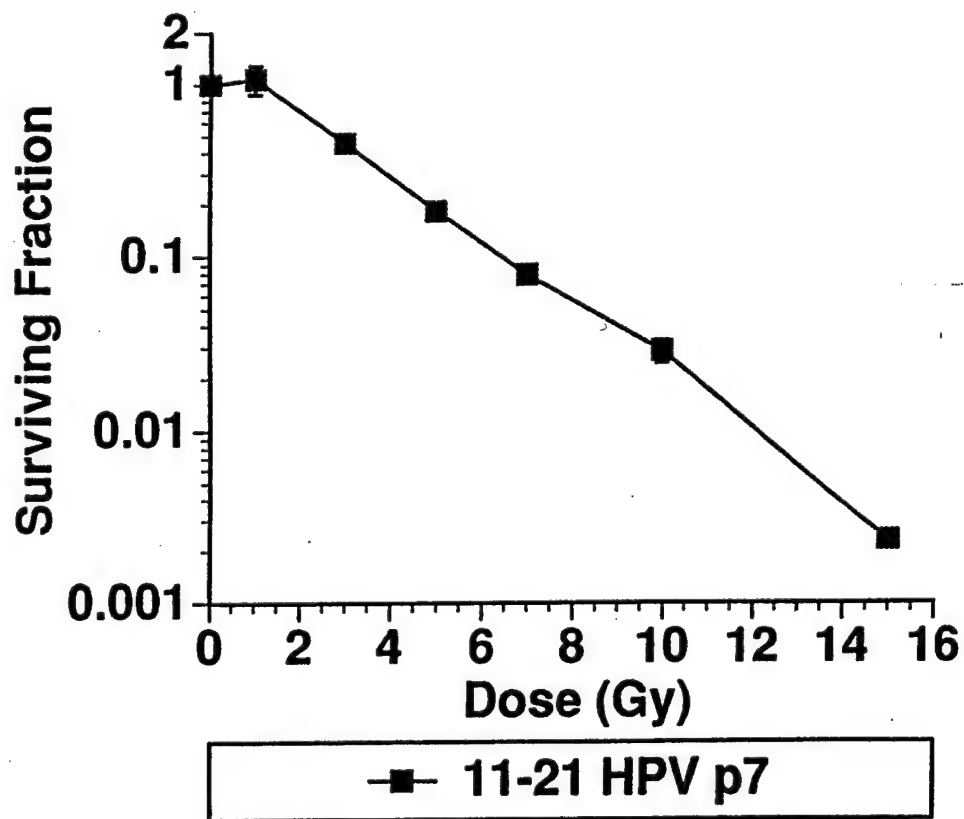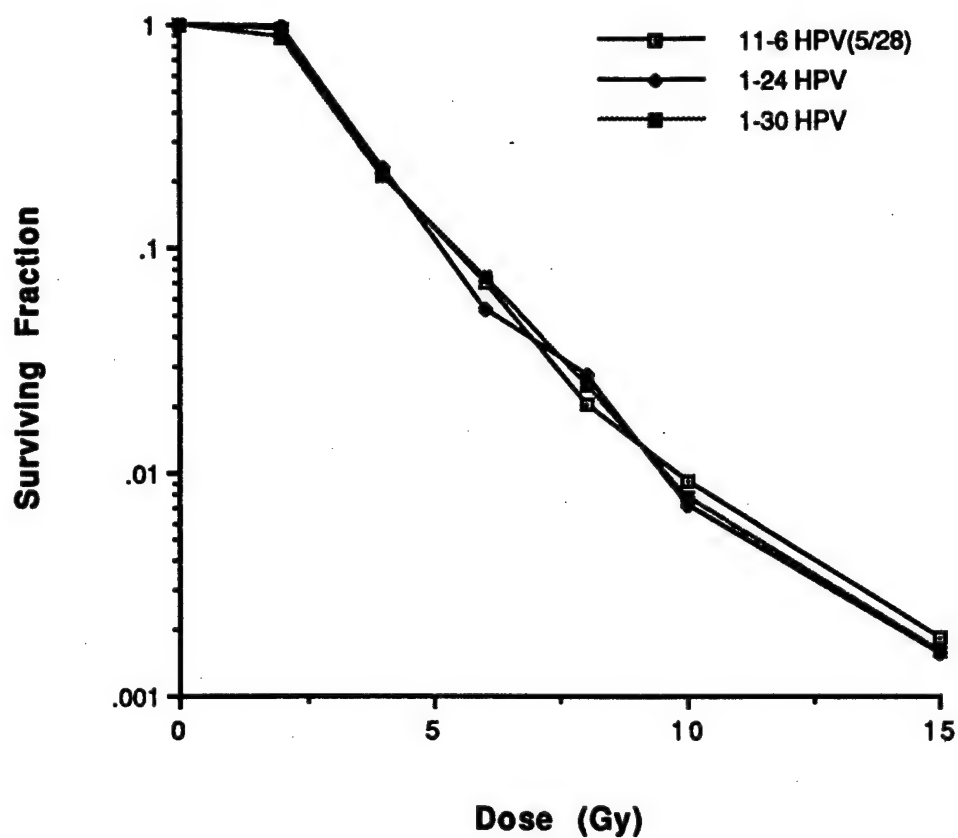
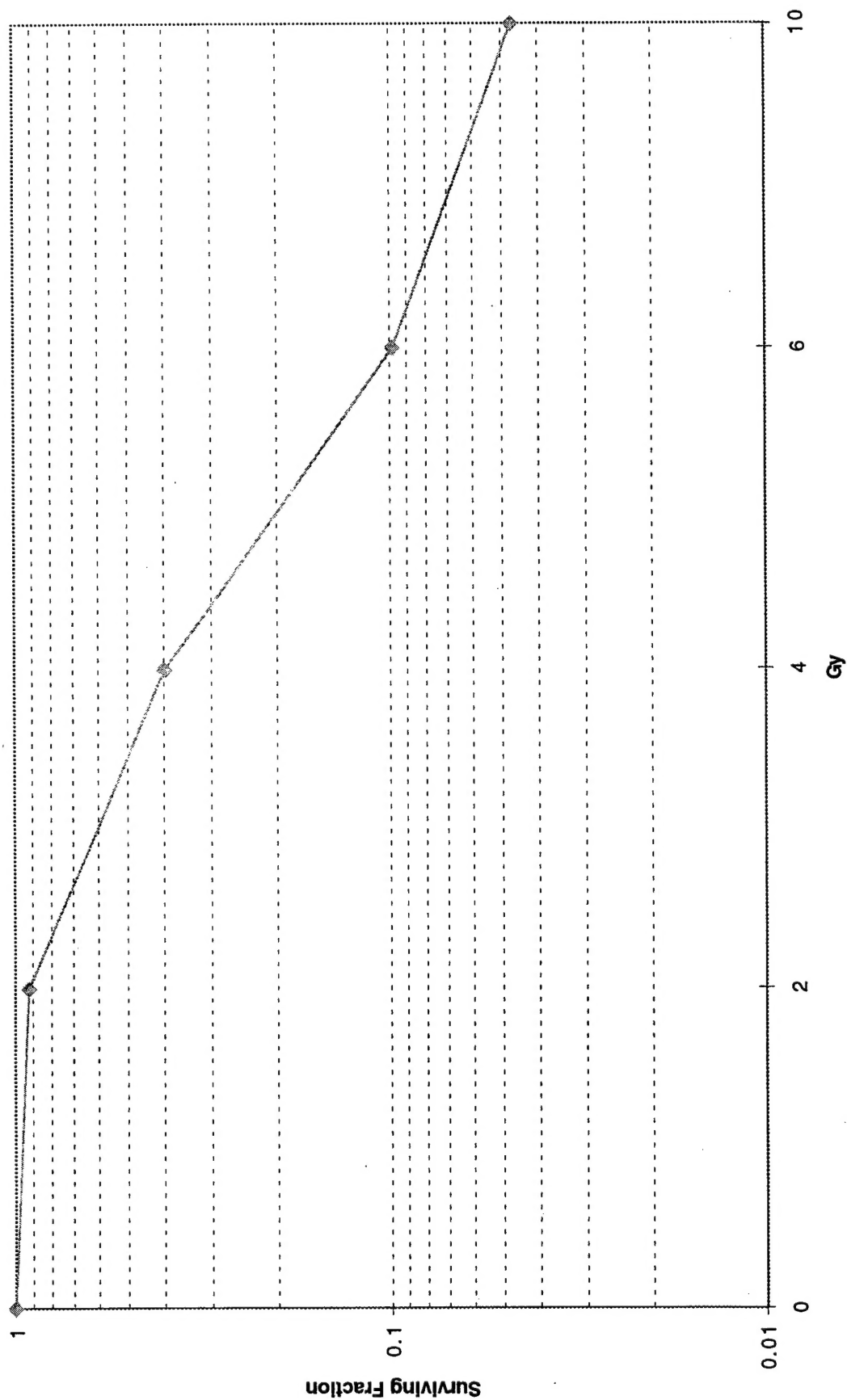## 11-6, 1-24 & 1-30 HPV Curve

Figure 1

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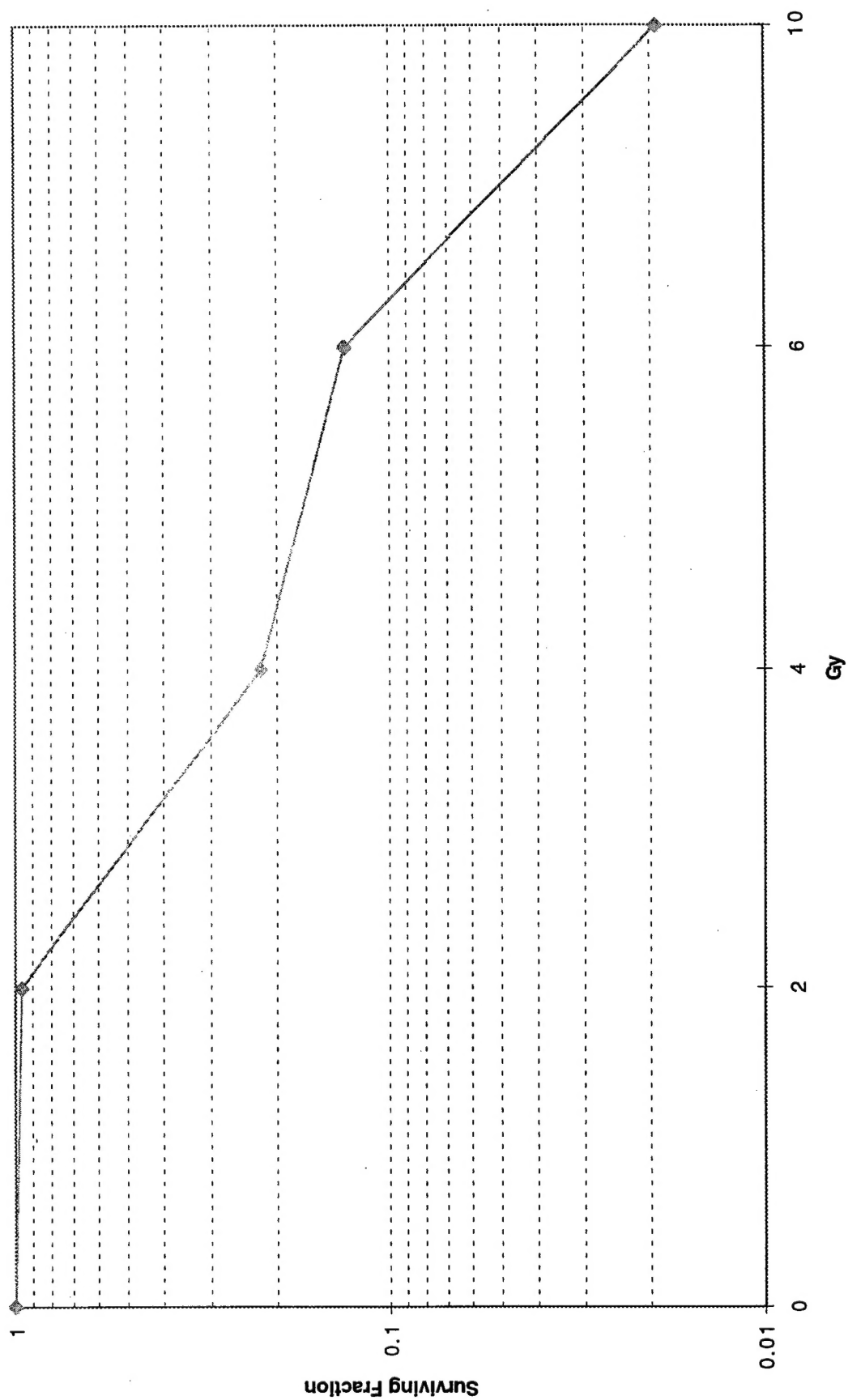11-21 E7, 3um

11-21 E7, 3 um

Figure 2

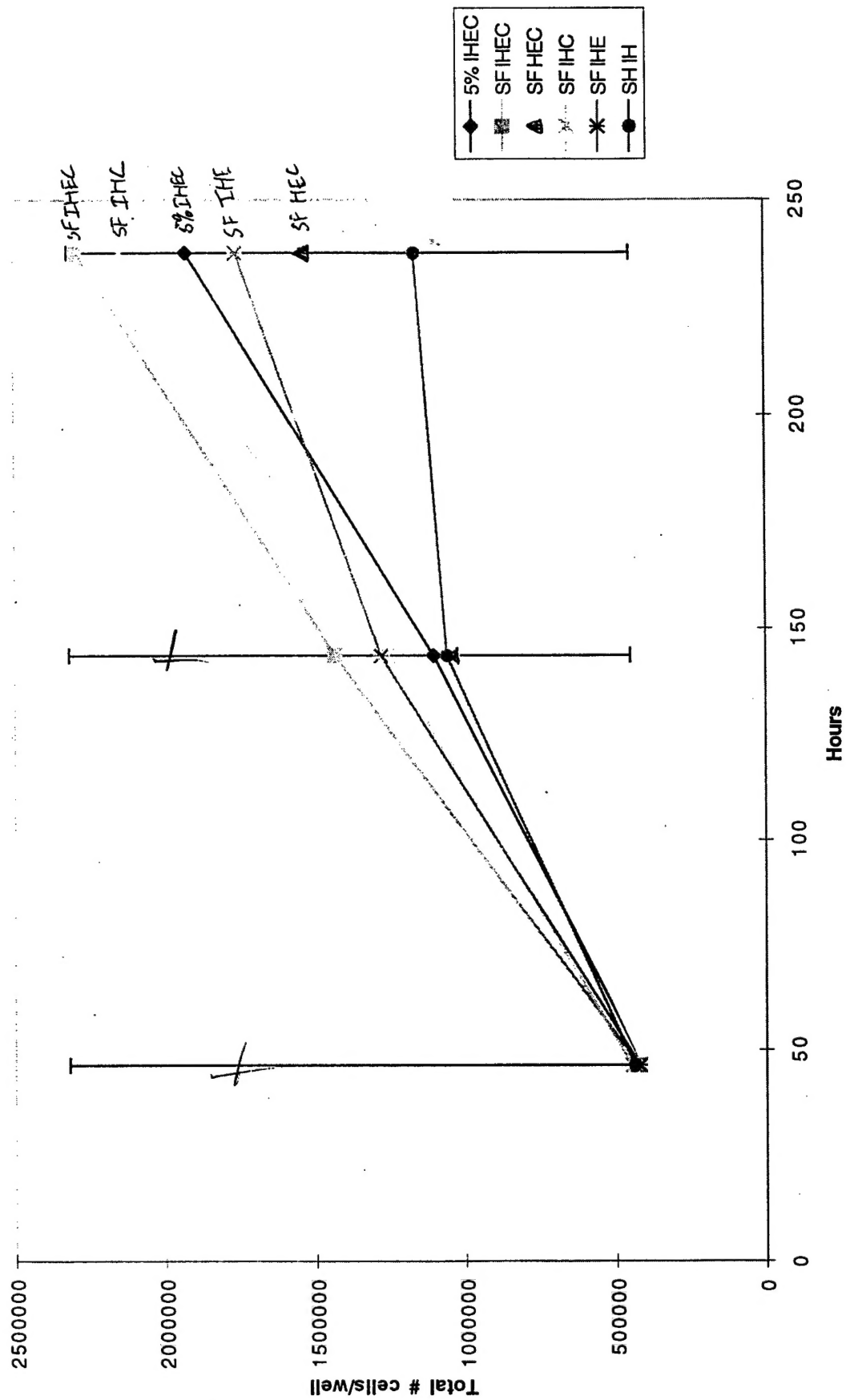# 11-6 E7, 3 um

Figure 3

11-21P5 E7P3

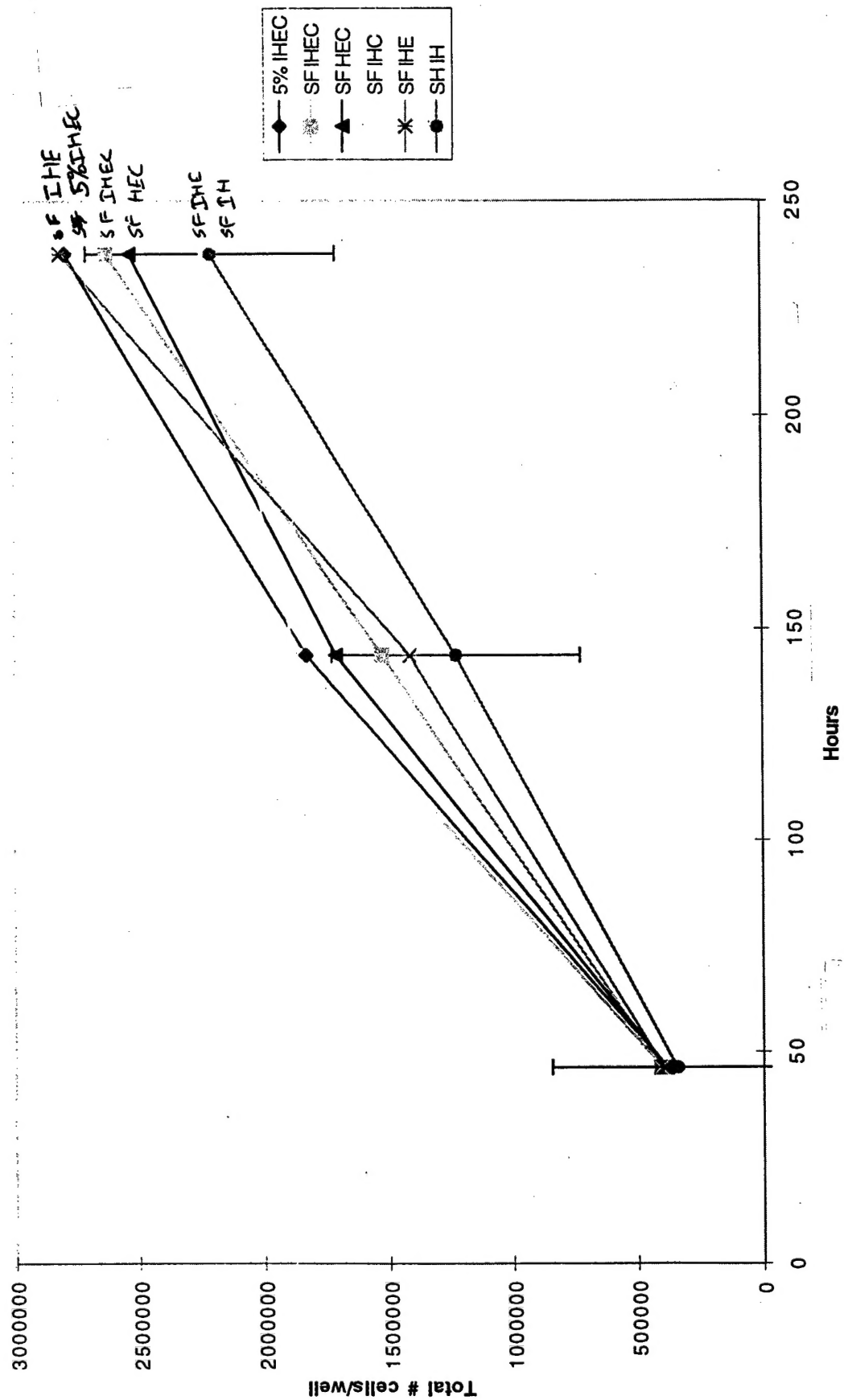Figure 4

Legend:
- 5% IHEC
- SF IHEC
- SF HEC
- SF IHC
- SF IHE
- SH IH

X-axis: Hours (0, 50, 100, 150, 200, 250)

Y-axis: Total # cells/well (0, 500000, 1000000, 1500000, 2000000, 2500000)

Handwritten labels: SF IHEC, SF IHC, 5% IHEC, SF IHE, SF HEC

11-6P5 E7P3

Figure 5